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Evidence for the Dark Repair of Ultraviolet Damage in Saccharomyces Cerevisiae Mitochondrial DNA (39065)

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The question of whether mitochondrial DNA is subject to dark repair, although studied in some detail has not been answered conclusively. Any attempt to investigate such repair is complicated by the fact that both mitochondrial and nuclear DNA are vulnerable to photochemical damage and it is difficult to distinguish between the effects on these two targets. The yeast cell is a useful subject for study because as a facultative anaerobe it is able to survive with or without functioning mitochondria. Hence, even though the mitochondrial DNA of a yeast cell may be rendered as nonsense, missing, or irreparably damaged, resulting in inoperable mitochondria, the cell is still able to survive in the form of a petite (*rho*$^-$) mutant (1). The survival of these two cellular DNAs may then be examined separately after ultraviolet damage by determining the overall fraction of survival in a given cell population which is dependent only upon the repair of nuclear DNA. In addition, mitochondrial damage may be evaluated by scoring from the total number of survivors those which appear as pure clones of petites and those colonies which appear sectored due to a mixed population of *rho*$^-$ mutants and normal (grande) cells. The petite phenotype may also arise from a nuclear mutation (1). Since the two (cytoplasmic and segregational petites) cannot be distinguished without genetic characterizations, no distinction has been made in the data presented here. However, the number of petites induced by ultraviolet irradiation may still be used to reflect mitochondrial DNA damage and repair since segregational petites have been shown to comprise a small percentage of the total petite population (2).

The question of whether dark repair occurs in mitochondrial DNA has been answered in part by Maroudas and Wilkie (3) and Moustacci and Enteric (4). Maroudas and Wilkie reported that the number of petites at a low dosage of ultraviolet light was reduced after a split dose versus a continuous dose. Only one dose, however, was investigated. Moustacci and Enteric reported that after ultraviolet irradiation followed by a period of liquid holding of diploid yeast in a non-nutritive media, an increase in the number of petites resulted upon the delayed plating of the yeast. This effect, although the opposite of Maroudas and Wilkie's finding, was explained on the basis of a degradative process of repair which predominated over the resynthesis step. Later work indicated that the ability to repair cytoplasmic DNA may depend upon the stage of growth in which yeast are irradiated (5). However, ultraviolet induced pyrimidine dimers in yeast mitochondrial DNA did not appear to be excised after a liquid holding period (6). Experiments with ultraviolet sensitive mutants derived from defects in chromosomal genes have indicated that any dark repair of yeast mitochondrial DNA would of necessity involve enzymes coded from both mitochondria and nuclear DNA (7). Moreover, Westergaard and co-workers (8–10) have demonstrated the induction by ultraviolet light of a mitochondrial DNA polymerase in Tetrahymena. Such a polymerase may function as a repair enzyme. The purpose of the present investigation was to demonstrate whether dark repair of mitochondrial DNA does occur in yeast, and to provide some understanding of the different results reported previously.

Materials and methods. Strain and growth conditions. A derivative of ATCC No. 4098 *Saccharomyces cerevisiae* var. *ellipsoideus* was used in all experiments. Cultures were grown in a shaking water bath at 30° in 1% yeast extract, 2% bacto-peptone, 1% glucose, and 1% pyruvate for 24–25 hr (early stationary phase) using an initial inoculum

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of 10^6 cells/ml. At early stationary phase minimum cell budding occurred and no cell clusters were seen.

Experimental procedure. Cells were harvested and washed three times at room temperature with 0.067 *M* phosphate buffer pH 7.0 and suspended in buffer to a concentration of 10^3 or 10^4 colony forming units per ml. Suspensions (20 ml) were irradiated in an open petri dish placed under a Gates Raymaster Lamp with filter, emitting radiation at 254 nm. The emitted dose was calibrated by the photohydrolysis of uranyl nitrate and oxalic acid (11). Immediately after irradiation samples were diluted in buffer and plated in subdued light while the remaining suspension at each dose was placed in a covered 50 ml flask on a shaking water bath in the dark at 30° for liquid holding. After the liquid holding period, samples were again withdrawn, diluted and plated. Dilutions were adjusted to produce approximately two hundred colonies per plate. Cell suspensions were spread on 1% yeast extract, 2% bacto-peptone, 1.5% agar (YEP) and either 1% glucose or 1% pyruvate as the carbon source. The introduction of 1% pyruvate as the sole carbon source was to select for the growth of grande colonies only. The use of a differential plating technique to select for grandes was first introduced by Ogur (12) in which lactate was used as the selective carbon source. For these experiments the selectivity of pyruvate was demonstrated by plating suspensions of 33 whole colony petites that had been identified from plates overlaid with tetrazolium chloride agar. Inoculation of petites onto plates of 1% glucose or 1% pyruvate YEP resulted in growth only on plates containing 1% glucose as the carbon source. Thus, only those yeast cells with viable mitochondria could utilize pyruvate and grow into visible colonies on 1% pyruvate plates. For liquid holding in caffeine, 10 ml of each suspension was added to a flask containing 12 mg of dry caffeine crystals to obtain a final concentration of 1.2 mg/ml caffeine. Solution of the crystals occurred immediately upon addition of liquid. Plates containing either 1% glucose or 1% pyruvate were incubated at 30° for 2 days or 3 days, respectively. In each experiment every dose

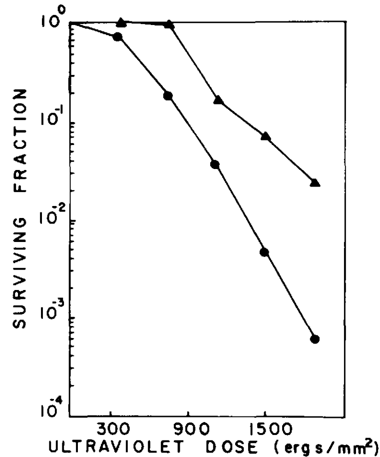

FIG. 1. The total survival of all colony types of *Saccharomyces cerevisiae* on 1% glucose YEP after ultraviolet irradiation followed by ● immediate plating, and ▲ delayed plating after 24 hr of liquid holding in 0.067 phosphate buffer, pH 7.0, 30°.

level and two unirradiated controls were plated in triplicate and the counts were averaged. All plates were scored for the number of grande, sectored and whole colony petites. The detection of petites was done according to the tetrazolium overlay procedure as described by Ogur (13). The average percentage of spontaneous petites (always 1%) which occurred among the unirradiated control colonies was subtracted from each set of data in which the percentage of petites is reported. The average increase in the number of colony forming units in the unirradiated controls after a period of liquid holding was approximately 5%. Each experiment discussed is one of at least three similar experiments.

Results. Recovery at high dose levels. The effects of immediate and delayed plating on the survival of yeast following ultraviolet irradiation are shown in Fig. 1. An increased survival due to delayed plating in this experiment is consistent with DNA repair but does not distinguish between nuclear and mitochondrial events. The effects of ultraviolet damage to mitochondrial DNA may be observed by the subsequent induction of petite mutants. In all the experiments discussed here the "% Mutants" refers to the percentage of petites present expressed as the percent of the unirradiated control

colonies. The control population did not vary significantly during the liquid holding period. Other workers have often expressed the induction of petites as the number of petites per survivors. Such an expression involves the quotient of two possible variables, the number of petites induced and the number of survivors at each dose level. This treatment may become confusing when the survival and mutation data are compared after immediate and delayed plating, because of the large fluctuations which may occur in the numbers of survivors and petites in the two experiments. In dosage ranges where the total cell survival approaches 100% after immediate and delayed plating, these two expressions of % Mutants do not vary considerably, and in the discussions here the same conclusions may be drawn from either treatment of the data. However, an expression of the number of petites per unirradiated control colonies which in fact is the survival fraction of petites appears to us to be a more straightforward treatment of the data.

Figure 2 shows that the production of petites when expressed as the log of % Mutants has a linear relationship to the ultraviolet dose. A theoretical extrapolation of these data indicates that 100% petites plus sectored colonies and petites only could be achieved at approximately 840 ergs/mm² and 975 ergs/mm² respectively, provided that complicating killing and repair effects could be set aside. The extrapolation therefore indicates the dose beyond which sufficient potential lethality may be inflicted to induce 100% petites. It does not state that we should expect 100% petites experimentally at these doses for two reasons. First, at dose levels of 1000 ergs/mm² less than one cell in a hundred survives the irradiated dose. It is probable that those cells which do survive have either received a lower dose from the random distribution of doses which occur throughout the cell population, or that the cell is very efficient in the repair of ultraviolet damage, both nuclear and cytoplasmic. In either case the percentage of petites appearing is not a true sampling of the actual petites induced because greater than 99% of the original population is not available for evaluation.

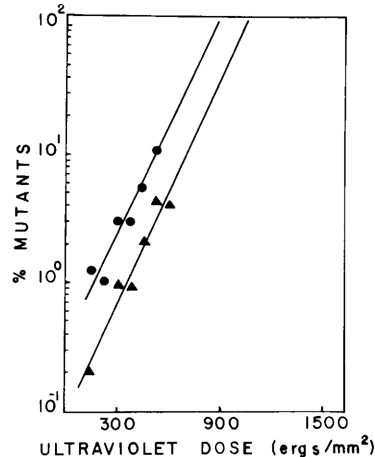

FIG. 2. The induction of petite mutants by ultraviolet light followed by immediate plating onto 1% glucose YEP: ● whole colony petites plus sectored colonies, ▲ whole colony petites. An extrapolation to 100% Mutants is achieved at 840 ergs/mm² for whole colony petites plus sectored colonies, and 975 ergs/mm² for whole colony petites.

Second, the survival curve upon immediate plating (Fig. 1) exhibits a repair shoulder which indicates that some repair of ultraviolet damage is possible between the time of immediate plating and cell replication. Assuming that cytoplasmic DNA is also subject to repair one would expect fewer petites than predicted from the extrapolated data after immediate plating. Having clarified this point, in spite of these limitations, the purpose of the extrapolation in Fig. 2 is to obtain a reasonable estimate of the dosage beyond which significant damage has occurred to mitochondrial DNA. From the combined data in Figs. 1 and 2, it can be concluded that irradiation $\geq$1500 ergs/mm² produced considerable lethal damage to both mitochondrial and nuclear DNA. Thus, any increase in the number of grande colonies at high dose levels after liquid holding should demonstrate repair of ultraviolet damage in both nuclear and mitochondrial DNA, since repair of all cellular DNA must be accomplished to achieve a normal colony state. In this instance a grande colony is defined as a clone of cells resulting from the ability of a parent cell or cells to replcate through the utilization of pyruvate as a carbon source, or from the ability of the colony to appear as a non-

TABLE I[a]

Ultraviolet dose (ergs/mm²)	Increase due to petites + sectored colonies (%)	Increase due to grandes (%)
375	0	100
750	1	99
1125	17	83
1500	27	73
1875	27	73

[a] The percentage contribution to the increase in cell survival from immediate to delayed plating on 1% glucose YEP. Delayed plating followed a period of liquid holding 24 hr in phosphate buffer, pH 7.0, 30°. Each percentage increase is the average increase calculated from three experiments.

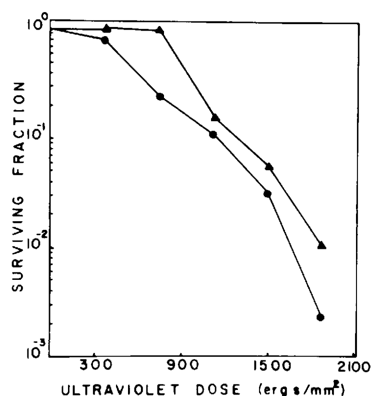

FIG. 3. The selective survival of grandes on 1% pyruvate YEP after ultraviolet irradiation followed by ● immediate plating, and ▲ delayed plating after 24 hr of liquid holding in 0.067 phosphate buffer, 0.067 *M* pH7.0, 30°. An increase in survival at ≥ 1500 ergs/mm² after delayed plating indicates dark repair of both nuclear and mitochondrial DNA.

petite after tetrazolium overlay on YEP 1 % glucose plates.

To test for an increase in the survival of grandes at high ultraviolet doses, parallel liquid holding experiments were performed in which yeast were spread onto two sets of plates, YEP with 1% glucose and YEP with 1% pyruvate. Grandes and petites were scored on plates containing 1% glucose by the use of the tetrazolium overlay procedure. An increase in survival after delayed plating at each dose level (Fig. 1) resulted from an increase in petites and grandes. The percent contribution from each colony type to produce an increase in the total number of colonies after liquid holding recovery was calculated using the data from three identical experiments. As shown in Table I, there was a net increase at high dose levels in the number of grande colonies after liquid holding. Although an increased contribution to survival was also seen from the numbers of petites and sectored colonies, the only valid measure here for repair of both cytoplasmic and nuclear DNA was the increase in grandes. The experiments in Fig. 3 indicate that the number of grande colonies on 1% pyruvate medium after ultraviolet irradiation was increased by delayed plating. Since Figs. 1 and 2 showed that both nuclear and mitochondrial damage would be expected at the higher dose ranges, it is presumed that the production of additional grande colonies observed on this selective medium reflected both mitochondrial and nuclear repair. The overall survival was somewhat better on pyruvate than on glucose perhaps because a slower growth rate permitted more time for repair.

Recovery at low doses. A more direct proof of the repair of mitochondrial DNA is the reduction in the total number of petites and sectored colonies after liquid holding. This phenomenon can be observed at low dose levels in which total cell survival remains high. This approach was used to demonstrate the photorepair of cytoplasmic DNA (14). Figure 4 shows experiments in which the effects of liquid holding were studied on the production of petites by low dose ultraviolet irradiation. The number of whole colony petites was plotted as the percent of the number of unirradiated control colonies on immediate and delayed plating. As shown, there was an overall decrease in this number after delayed plating. It is important to note that the data when expressed as petites/unirradiated control revealed a maximum in the % petites which thereafter declined sharply. This decline is due to the large increase in cell killing above 525 ergs/mm² (see Fig. 1). Therefore, although the actual proportion of petites increased greatly among the survivors at each increased dose level, the total

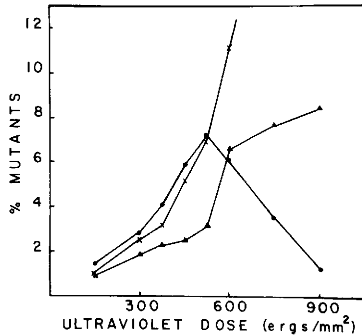

FIG. 4. The induction of whole colony petites at low doses of ultraviolet light: ● immediate plating; ▲ delayed plating after 24 hr of liquid holding in phosphate buffer, pH 7.0, 30°; × delayed plating after 24 hr of liquid holding as above with the addition of 1.2 mg/ml of caffeine. A decrease in the number of petites after delayed plating up to 525 ergs/mm² suggests dark repair of mitochondrial DNA. The lack of a marked decrease in % mutants after a period of liquid holding in caffeine indicates an inhibition of mitochondrial DNA repair by caffeine.

number of petites surviving was reduced. By focusing on an area of the survival curve where the total survival remains high, meaningful fluctuations in the total number of petites could be observed. Furthermore, although the total cell survival increases slightly after 24 h of liquid holding at 525 ergs/mm², a net decrease in the number of whole colony petites occurred. A similar decline in the number of whole colony petites plus sectored colonies was seen. However, since the exact scoring of sectored colonies was often doubtful and while whole colony petites were easily identified (completely white colonies after tetrazolium overlay) all data shown in Fig. 4 represent the percent of whole colony petites. Using the data from three similar experiments a calculation was made using the Student's t to test the significance of the decrease in the number of petites due to liquid holding for doses up to 525 ergs/mm². Although the percentage decrease is small it is highly reliable (see Table II).

TABLE II

Ultraviolet dose (ergs/mm²)	Student's t test (df = 2)	P*
300	1.6	$<.30$
375	5.2	$<.05$
450	20.6	$<.01$
525	9.9	$<.01$

Calculation of the Student t to determine the significance of the decrease in % Mutants (whole colony petites) at low doses of ultraviolet irradiation from immediate to delayed plating. A typical induction curve showing a decrease in % Mutants after delayed plating is shown in FIG. 4.

* P is the probability of having this t value by chance.

Caffeine has been shown to inhibit the liquid holding recovery of stationary phase yeast (4) and to have only a slight effect on the liquid holding recovery of irradiated dividing cells. Thus, it has been suggested that caffeine inhibits primarily the excision repair of irradiated DNA in yeast. A similar repair inhibition by caffeine in yeast mitochondrial DNA should result in an increase of petite mutants after ultraviolet irradiation and subsequent liquid holding in caffeine. To verify further that the decrease in the population of petites after liquid holding is due to repair of cytoplasmic DNA, a survival experiment was done with and without the presence of caffeine in the liquid holding cell suspension. Caffeine reduced the total cell survival after 24 hr of liquid holding due to the inhibition of nuclear DNA repair. In an unirradiated control with caffeine no petites were induced except for a low level (<1%) of spontaneous petites which also appeared in controls without caffeine. A marked decrease in the number of petites did not occur when caffeine was added to the liquid holding buffer. This seems to demonstrate more clearly that the reduction in the number of petites at low ultraviolet doses after liquid holding without caffeine may be attributed to the repair of mitochondrial DNA.

Discussion. Interpretation of the survival data can be divided into two areas: high and low levels of cell survival. In the high dose area the recovery of cells after liquid holding involves a large population of cells. For example, at 1875 ergs/mm² in Fig. 1 the increase in cell survival after 24 hr of liquid holding showed a thirtyfold increase in survival over that observed with imme-

diate plating. Combining the data in Figs. 1 and 2 one might conclude that lethal damage to all cellular DNA occurs at high ultraviolet dose levels. If only nuclear DNA in a cell is repaired, then new colonies after liquid holding would appear as petites; however, if only mitochondrial DNA is repaired the cells will not grow because nuclear DNA survival is a requisite for cell survival. It is not surprising, therefore, that an increase in the number of petites may be seen after liquid holding at high doses as shown by our data (Table I) and by Moustacci and Enteric (4), due to the repair of extensive nuclear damage. However, this says very little about DNA repair in mitochondria. At high doses it appears to be more significant to determine if an increase in the number of normal colonies results after liquid holding which testifies to the fact that both systems have undergone dark repair of DNA. Such an increase was demonstrated in these experiments after delayed plating on 1% glucose and 1% pyruvate media.

In the low dose area the range of cells surviving after 24 hr of liquid holding is much more restricted. The changes in cell survival on delayed plating are much less dramatic so that fluctuations in the number of petites induced are easier to interpret. Hence, the decrease seen in the number of petites at 24 hr of liquid holding must point to a shift in the population of survivors from petites to grandes. It is not likely that this decrease in petites is due to their selective cell death during the period of liquid holding, because we are examining a population fluctuation within the shoulder region of the survival curve where very little killing is evident. The effect of caffeine in preventing this decrease after liquid holding is further evidence against cell death since this agent is presumed to act as a repair inhibitor.

This reduction in the number of petites induced at low doses and the overall increase in the number of grandes at high doses of ultraviolet light indicates strongly that ultraviolet damage to mitochondrial DNA is subject to dark repair resulting in a measurable recovery from damage.

Summary. Evidence for the dark repair of ultraviolet damage to yeast mitochondrial DNA has been observed. The ultraviolet dose necessary to inflict significant damage to both nuclear and mitochondrial DNA was determined. Cell survival at large doses of ultraviolet light was observed after immediate and delayed plating of yeast onto 1% pyruvate and 1% glucose media. In the highly lethal dose ranges of irradiation an increase in the number of normal colonies appeared after a period of liquid holding and delayed plating. This increase, demonstrated separately on 1% glucose and 1% pyruvate media suggested that the repair of both mitochondrial and nuclear DNA had occurred. After low doses of ultraviolet light an actual decrease in the number of petite survivors was seen after delayed plating, even though the total number of survivors increased. When a known repair inhibitor, caffeine, was added to the liquid holding buffer prior to the delayed plating of yeast, a marked decrease in the number of petites did not occur after delayed plating. Therefore, the decrease in the number of petite survivors after delayed plating following low doses of ultraviolet light is attributed to the repair of yeast mitochondrial DNA.

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